Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2457–2461, May 1983
Biochemistry

Folding autonomy of the kringle 4 fragment of human plasminogen

(protein folding/protein evolution)

MÁRIA TREXLER AND LÁSZLÓ PATTHY

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary, H-1502

Communicated by Emil L. Smith, January 13, 1983

ABSTRACT **Kringle 4, an 88-residue plasminogen fragment carrying a lysine-binding site, loses its affinity for lysine-Sepharose upon reductive cleavage of its disulfide bridges. Aerobic incubation of the reduced, denatured fragment results in the rapid restoration of the disulfide bonds with concomitant recovery of lysine-Sepharose affinity. The ability of the unfolded fragment to regain its native conformation suggests that the kringle structure is an autonomous folding domain. During refolding of kringle 4 the native disulfide bonds, Cys^{22} Cys^{62} and Cys^{50} Cys^{74}, appear first. The folding intermediate possessing these two disulfide bridges already binds to lysine-Sepharose, indicating that the third native bridge, which in native kringle 4 connects residues Cys^{1} and Cys^{79}, is not essential for the maintenance of the biologically active conformation of kringle 4. Comparison of the sequences of human prothrombin, urokinase, and plasminogen kringles revealed that the residues surrounding the Cys^{22} Cys^{62} and Cys^{50} Cys^{74} bridges constitute the most conservative segments of kringles, whereas the residues neighboring the Cys^{1} Cys^{79} bridge are not highly conserved. We propose that conservation of various residues in the different kringles reflects their importance for the folding autonomy of kringles.**

Plasminogen, prothrombin, and urokinase, unlike the proenzymes of pancreatic serine proteases, possess large protein extensions at the amino-terminal end of the homologous protease part (1–4). These nonprotease segments are important for the biological specificity and control of plasmin, thrombin, and urokinase because they are involved in interactions that regulate plasminogen activation and plasmin-catalyzed fibrinolysis (5) or control the prothrombin–thrombin conversion (6). Thus, the nonprotease regions participate in the Ca^{2+}-mediated binding of prothrombin to phospholipid membranes, in the association of prothrombin with factor Va (7, 8), and in the interactions of plasmin(ogen) with fibrin, α_2-antiplasmin, and ω-aminocarboxylic acids (9–11).

The multiplicity of binding functions associated with the nonprotease segments of prothrombin, plasminogen, and urokinase is reflected in the structure of these proteins inasmuch as these regions are divided into discrete structural–functional units. In the case of plasminogen five triple-loop, three-disulfide-bridge "kringles" are discernible in the nonprotease segment (1). The five structural units display identical gross architecture and remarkable sequence homology, making the conclusion inescapable that these units arose as a result of 5-fold internal repetition of the same gene piece (12, 13). The amino-terminal part of prothrombin also contains two kringles, closely homologous to those of plasminogen (2, 3); the kringles of plasminogen and prothrombin are in fact more closely related than their protease parts (12). Urokinase was also shown recently to possess a kringle structure that shows extensive homology with the prothrombin and plasminogen kringles (4).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The kringles are independent structural domains. By limited proteolysis, a technique suitable to detect structural domains of proteins, it was shown that both prothrombin and plasminogen may be dissected at the boundaries separating kringles, while leaving the kringles intact (1, 2). Circular dichroism studies on prothrombin and its proteolytic fragments showed that little alteration occurs in the structure of kringles after isolation of the fragments, suggesting that in prothrombin the kringles exist as distinct, noninteracting structural domains (14). Differential scanning calorimetry of plasminogen, prothrombin, and their proteolytic fragments revealed that the ΔH value of the thermal transition of intact prothrombin or plasminogen is the sum of the ΔH value of the thermal transitions of the individual fragments, indicating that the kringles exist as independent domains (15, 16).

The kringles also are independent functional domains. Thus, isolated kringle 1 and kringle 4 fragments of plasminogen retain their capacity to bind ω-aminocarboxylic acids (1, 17), isolated kringle 5 binds to benzamidine-Sepharose (18), and fragment 2 of prothrombin, corresponding to one of the prothrombin kringles, carries an intact binding site for factor Va (8, 19).

The structural and functional independence of kringles suggested to us that these small triple-loop "miniproteins" also may be autonomous with respect to folding. In the present investigation we have found that the reduced, denatured kringle 4 fragment of human plasminogen regains its native, biologically active conformation when exposed to nondenaturing conditions. Our results suggest that the high degree of conservation of large segments of kringles is due to the importance of the conserved sequences for the folding autonomy of kringles.

METHODS

Kringle 4 was prepared by limited proteolysis of human plasminogen as described (11). The concentration of kringle 4 was determined by using an ε_{280} of 3.1×10^4 $M^{-1} \cdot cm^{-1}$ (20) and a molecular weight of 10,000 (1).

The biological activity of kringle 4 was assessed by its binding to lysine-Sepharose 4B (11, 20). Affinity of kringle 4 for lysine-Sepharose was assayed at 25°C by column chromatography; the size of the column was selected such that the sample exhausted about 20% of the column capacity. The lysine-Sepharose 4B column was equilibrated with 0.1 M ammonium bicarbonate buffer (pH 8.0), and the sample to be assayed was applied in the same buffer. After loading the sample onto the column, it was washed with 3 bed vol of buffer to remove any kringle 4 that lost affinity for lysine-Sepharose, and then kringle 4 that was bound to the column was eluted with buffer containing 10 mM ε-aminocaproic acid. The amount of kringle 4 present in the bound and nonbound fractions was determined spectrophotometrically.

Unfolding of kringle 4 (0.5 mM) was performed by incubating the protein in 0.1 M Tris·HCl/5 mM EDTA/6 M guanidinium chloride, pH 8.0, at 25°C for 60 min. Reduction of the disulfide bonds was achieved by incubating kringle 4 (0.5 mM)

in 0.1 M Tris·HCl/5 mM EDTA/6 M guanidinium chloride, pH 8.0, buffer containing 5% (vol/vol) 2-mercaptoethanol or 100 mM dithiothreitol at 25°C for 30–120 min. To determine reduction of disulfide bonds, aliquots of the reaction mixture containing the reduced, denatured protein were alkylated with iodoacetate, used in 5-fold molar excess over thiol groups. Salts and reagents were removed by gel filtration on Sephadex G-25 columns equilibrated with 0.1 M ammonium bicarbonate and the alkylated protein was lyophilized. After acid hydrolysis the S-carboxymethylcysteine content of the protein was determined by amino acid analysis.

Refolding of reduced, denatured kringle 4 was initiated by removal of the reducing and denaturing agents. The reaction mixture containing reduced denatured kringle 4 was gel-filtered at 25°C on Sephadex G-25 columns equilibrated with refolding buffer (0.1 M ammonium bicarbonate buffer at pH 8.0, unless otherwise indicated). During aerobic refolding the refolding mixture (0.05 mM for kringle 4) was allowed to stand at 25°C and at intervals aliquots (containing 2 mg of kringle 4) were withdrawn to follow the recovery of the biological activity of kringle 4 by determining the fraction of molecules that regained affinity for lysine-Sepharose 4B. Aliquots (containing 2 mg of kringle 4) also were taken for alkylation with iodoacetate (final concentration, 50 mM) to trap folding intermediates. The alkylated protein was gel-filtered on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate and was lyophilized. The S-carboxymethylcysteine and half-cystine content of the samples was determined by amino acid analysis. Portions of the S-carboxymethylated samples were subjected to isoelectric focusing to follow the progress of refolding by separation of the folding intermediates.

Refolding also was performed in the presence of mixtures of oxidized and reduced glutathione (21).

Analytical isoelectric focusing on polyacrylamide slab gels was used to separate the alkylated folding intermediates of kringle 4. The gel (1.2 mm thick) contained 8% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 12% (wt/vol) sucrose, 5% (vol/vol) Ampholine, pH 3.5–10 (LKB), and 0.15% ammonium persulfate. Lyophilized, salt-free samples of kringle 4 were dissolved in deionized water at a concentration of 0.6 mM and 5-μl aliquots were applied to the slab gel. The electrode strips were soaked in 1 M NaOH (cathode) and 1 M phosphoric acid (anode) and electrophoresis was run at 10 W at 4°C for 240 min. The gels were immersed in 20% trichloroacetic acid overnight, stained for 3 hr with 0.1% Coomassie blue in 20% trichloroacetic acid, and destained by washing with 10% acetic acid.

In the case of experiments aimed at determining the structure of the two-disulfide-bridge folding intermediate, refolding and alkylation were performed as in the analytical studies, except that 30–50 mg of kringle 4 was used to prepare the S-carboxymethylated folding intermediate. Hydrolysis of the reduced, carboxymethylated folding intermediate with trypsin was performed at 37°C for 24 hr in 0.1 M ammonium bicarbonate buffer (pH 8.0). The reaction mixture contained the folding intermediate (10 mg/ml) and trypsin at an enzyme-to-substrate ratio (wt/wt) of 1:50. Tryptic peptides of kringle 4 were purified by high-voltage paper electrophoresis at pH 1.9 or pH 6.5 by using Whatman 3 MM papers as described (11).

Peptides and proteins were hydrolyzed in 6 M HCl at 110°C for 24 hr and the composition of the acid hydrolysates was determined by amino acid analysis on a Biotronik LC 2000 analyzer.

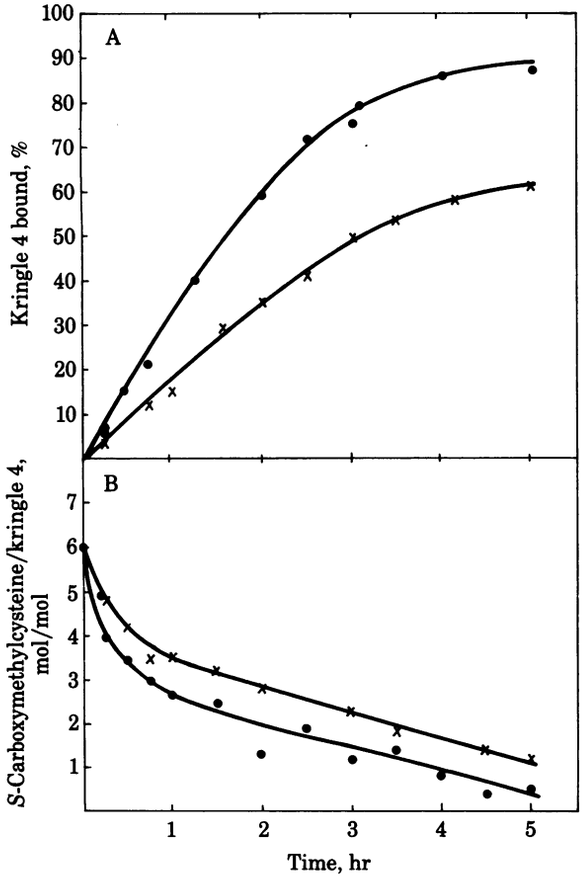

FIG. 1. (*A*) Recovery of lysine-Sepharose affinity of reduced, denatured kringle 4 during aerobic incubation in 0.1 M ammonium bicarbonate (pH 8.0) at 25°C in the absence (x) and presence (●) of 10 mM ε-aminocaproic acid. (*B*) Disappearance of free sulfhydryl groups of reduced denatured kringle 4 during aerobic incubation in 0.1 M ammonium bicarbonate (pH 8.0) at 25°C in the absence (x) and presence (●) of 10 mM ε-aminocaproic acid. During the course of refolding aliquots were alkylated with iodoacetate to block free sulfhydryl groups and the S-carboxymethylcysteine content of the samples was determined by amino acid analysis.

RESULTS

Autonomous Refolding of Reduced, Denatured Kringle 4. Exposure of kringle 4 to denaturing conditions, by incubation

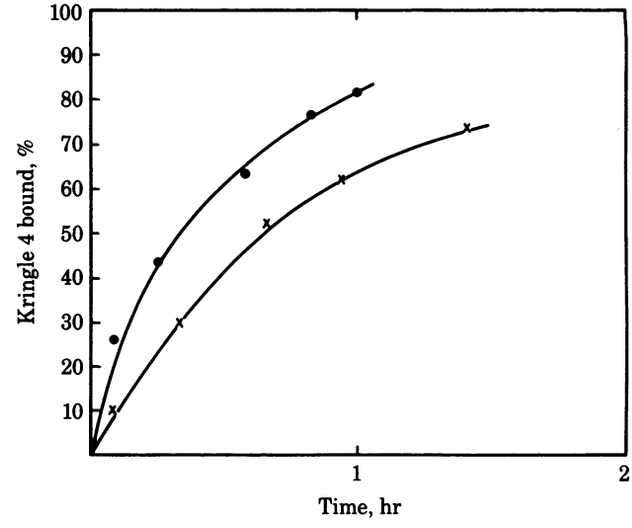

FIG. 2. Recovery of lysine-Sepharose affinity of reduced, denatured kringle 4 at 25°C in 0.1 M ammonium bicarbonate (pH 8.0) containing 0.1 mM oxidized and 1 mM reduced glutathione. Refolding was performed in the absence (x) and presence (●) of 10 mM ε-aminocaproic acid.

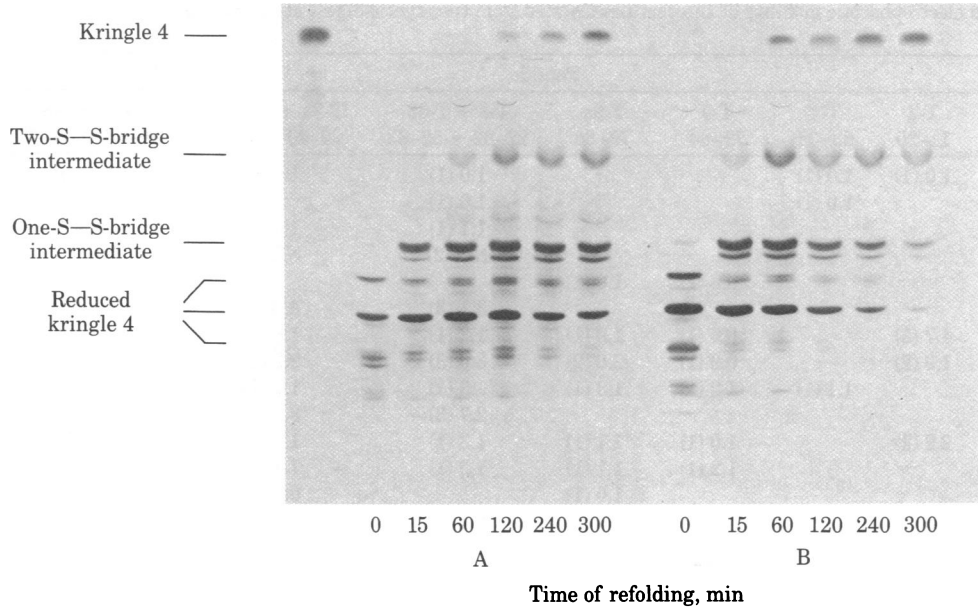

FIG. 3. Pattern of folding intermediates at various times during refolding of kringle 4. Refolding was performed in the absence (*A*) and presence (*B*) of 10 mM ε-aminocaproic acid as described in the legend to Fig. 1 and the *S*-carboxymethylated folding intermediates were resolved by isoelectric focusing.

in 0.1 M Tris·HCl/5 mM EDTA/6 M guanidinium chloride, pH 8.0, did not abolish the affinity of the fragment for lysine-Sepharose; the fragment was found to bind normally to lysine-Sepharose after removal of the denaturing agent. However, if the disulfide bridges of kringle 4 were cleaved with reducing agents during denaturation, the reduced, denatured kringle 4 fragment was not bound to the lysine-Sepharose column when assayed immediately after removal of reducing and denaturing agents.

On prolonged incubation of reduced, denatured kringle 4 under aerobic conditions, the lysine-Sepharose affinity was recovered progressively with a half-time of 3 hr, concomitant with the disappearance of the free sulfhydryl groups (Fig. 1). When refolding was performed in the presence of a mixture of oxidized and reduced glutathione (0.1 and 1.0 mM, respectively) the lysine-Sepharose affinity was regained with a half-time of 40 min (Fig. 2).

Recovery of lysine-Sepharose affinity and disappearance of free sulfhydryl groups of reduced denatured kringle 4 were both accelerated if the aerobic refolding buffer contained ε-aminocaproic acid (Fig. 1); the half-time of regain of biological activity was decreased from 180 to 90 min. The folding pathway was not altered significantly by the presence of ε-aminocaproic acid inasmuch as the same intermediates were discernible as those observed when refolding was carried out in the absence of ε-aminocaproic acid (Fig. 3). (Note that reduced, alkylated kringle 4 has about three isoelectric-point variants. Another feature worthy of mention is that reduced, alkylated kringle 4 and single-disulfide-bridge intermediates are stained more intensely than the two-disulfide-bridge intermediate and native kringle 4.)

The influence of ligand on the rate of refolding also was evident when refolding was performed in the presence of oxidized and reduced glutathione (Fig. 2) with the half-time of regain of biological activity being decreased from 40 to 20 min.

Structure of the Two-Disulfide-Bridge Folding Intermediate. Isoelectric focusing of refolding mixtures of kringle 4 re-

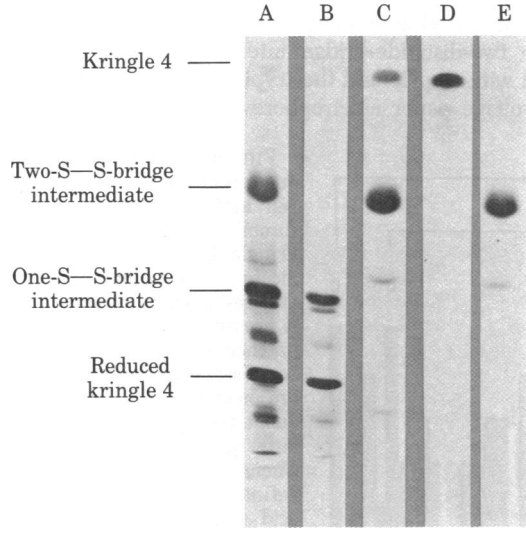

FIG. 4. Isoelectric focusing analysis of alkylated folding intermediates separated by lysine-Sepharose 4B affinity chromatography and ion-exchange chromatography. (*A*) Starting sample; (*B*) proteins not bound to lysine-Sepharose; (*C*) proteins bound to lysine-Sepharose. The proteins bound to lysine-Sepharose were chromatographed on QAE-Sephadex, as shown in Fig. 5, and kringle 4 was recovered in fractions not adsorbed to the ion-exchange resin (*D*). (*E*) The two-disulfide-bridge intermediate was eluted from the QAE-Sephadex column with 0.3 M ammonium bicarbonate.

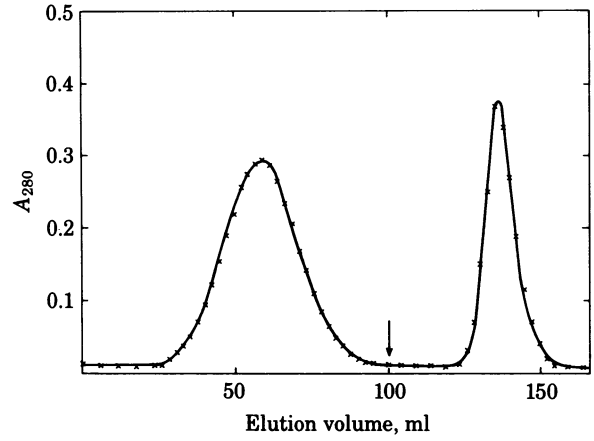

FIG. 5. Separation of kringle 4 and two-disulfide-bridge folding intermediate by ion-exchange chromatography. Chromatography was performed on a QAE-Sephadex A 50 (1.5 × 12 cm) column equilibrated with 0.1 M ammonium bicarbonate and kringle 4 was eluted with the same buffer (see Fig. 4*D*). The two-disulfide-bridge intermediate was eluted with 0.3 M ammonium bicarbonate (see Fig. 4*E*). The arrow marks the start of elution with 0.3 M ammonium bicarbonate.

Table 1. Amino acid composition and electrophoretic mobility of the peptides isolated from the tryptic digest of the two-disulfide-bridge intermediate

	Peptide							
Amino acid	T-1 −3–10	T-2 11–20	T-4 33–35	T-9 79–84	T-9a 79–86	T-3 + T-6b 22–32 + 52–63	T-3a + T-6 + 7 22–25 + 52–70	T-5 + T-8 36–51 + 71–77
Lysine		1.0 (1)	1.0 (1)			1.0 (1)	1.0 (1)	1.2 (1)
Histidine	1.0 (1)		1.0 (1)			1.0 (1)		
Arginine	1.0 (1)					1.1 (1)	1.0 (1)	1.0 (1)
Tryptophan						ND (2)	ND (1)	ND (1)
S-Carboxymethylcysteine	1.0 (1)			0.8 (1)	1.0 (1)			
Aspartic acid	2.3 (2)					3.1 (3)	4.2 (4)	4.3 (4)
Threonine		4.7 (5)		0.9 (1)	1.0 (1)	1.2 (1)	1.8 (2)	2.3 (2)
Serine	1.1 (1)	1.9 (2)		0.9 (1)	2.0 (2)	3.0 (3)	2.2 (2)	
Glutamic acid	2.4 (2)		1.1 (1)	1.2 (1)	1.3 (1)	1.3 (1)	1.2 (1)	2.0 (2)
Proline						2.7 (3)	3.0 (3)	2.1 (2)
Glycine	2.4 (2)	2.2 (2)		1.0 (1)	1.1 (1)	1.3 (1)	1.3 (1)	1.4 (1)
Alanine				1.2 (1)	1.1 (1)	1.0 (1)	1.1 (1)	1.3 (1)
Valine	1.0 (1)				1.0 (1)		0.9 (1)	
Methionine						0.6 (1)		0.7 (1)
Leucine								2.0 (2)
Tyrosine	2.2 (2)							2.8 (3)
Phenylalanine						0.8 (1)	1.0 (1)	
Half-cystine						+ (2)	+ (2)	+ (2)
Mobility in								
pH 1.9	0.73	0.76	1.56	0.44	0.40	0.81	0.70	0.64
pH 6.5	−0.11	0.42	0.77	−0.63	−0.52	0.2	0.12	0.08

Peptides are numbered consecutively from the amino-terminal end of kringle 4 (11). Numbering of amino acid residues (shown below each peptide) starts at the first half-cystine residue of kringle 4 to facilitate comparison of the five closely homologous kringles (1); therefore, the numbers are not identical with the numbering of amino acid residues of plasminogen. Electrophoretic mobilities are expressed relative to alanine and arginine in pH 1.9 and pH 6.5 electrophoresis, respectively. Numbers in parentheses are residue values based on sequence (1). ND, not determined. +, Trace.

vealed that there is only a single band corresponding to two-disulfide-bridge intermediate and a single band having three disulfide bonds (Fig. 3). To identify which of the six cysteines are present in the two-disulfide-bridge intermediate as free cysteines, kringle 4 (30 mg) was refolded for 120 min in the presence of ε-aminocaproic acid (10 mM) and was alkylated with iodoacetate to block the free sulfhydryl groups (see *Methods*). The refolding mixture, containing fully reduced kringle 4 and proteins with one, two, and three disulfide bonds (Fig. 4*A*), was applied on a lysine-Sepharose 4B column. The fully reduced and one-disulfide-bond forms of kringle 4 were not bound to the affinity column (Fig. 4*B*), whereas both the two-disulfide-bridge intermediate and the three-disulfide-bond protein (i.e., refolded kringle 4) were recovered in the fraction bound to lysine-Sepharose (Fig. 4*C*). Kringle 4 and the two-disulfide-bond intermediate were separated by chromatography on a QAE-Sephadex A 50 column (12 × 1.5 cm) equilibrated with 0.1 M ammonium bicarbonate (Fig. 5). Kringle 4 was recovered in the fraction not retained by the ion-exchange resin (Fig. 4*D*) and the two disulfide-bond intermediate was eluted from the column with 0.3 M ammonium bicarbonate (Fig. 4*E*).

The two-disulfide-bridge intermediate (Fig. 4*E*) was digested with trypsin and the tryptic peptides were isolated by high-voltage paper electrophoresis. Analysis of the peptides

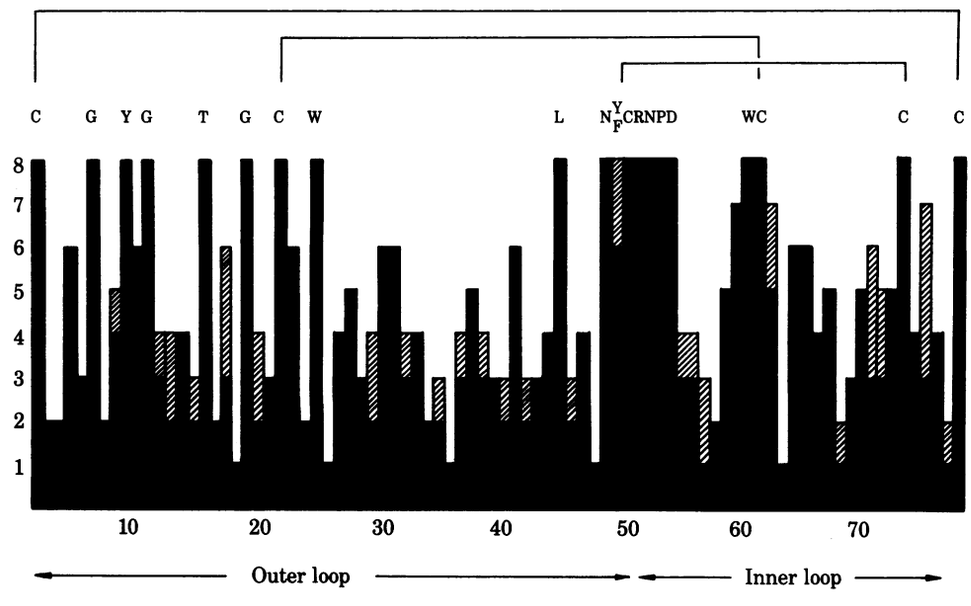

FIG. 6. Comparison of the sequences of the five human plasminogen kringles, the two human prothrombin kringles, and the urokinase kringle (1, 3, 4). The solid columns indicate the maximal number of sequences in which the same amino acid is present at the given position. The hatched columns represent the number of sequences in which residues that are chemically similar to the most frequent residue are present. The one-letter notation for amino acids is used. Chemically similar amino acids are defined as: lysine = arginine; aspartic acid = glutamic acid; asparagine = glutamine; serine = threonine; glycine = alanine; isoleucine = leucine = valine; and phenylalanine = tyrosine = tryptophan. Amino acid deletions or insertions were taken into consideration as residues not identical with any of the amino acids. The disulfide bridges and the residues conserved in the eight kringles are shown in the upper part of the figure.

showed that the intermediate has two native bridges, Cys^{50} Cys^{74} and Cys^{22} Cys^{62}, and residues Cys^{1} and Cys^{79} are present as *S*-carboxymethylcysteines (Table 1), indicating that formation of the Cys^{1} Cys^{79} bond is the last step of kringle 4 refolding. The ability of the two-disulfide-bridge intermediate to bind to lysine-Sepharose indicates that formation of the Cys^{1} Cys^{79} disulfide bridge is not essential for biological activity.

DISCUSSION

The Kringle Structure Is an Autonomous Folding Domain. In the last decade refolding studies on protein fragments have established that the distinct structural domains of multidomain proteins fold independently. Thus, isolated domains of immunoglobulins, plasma albumin, and ovomucoid were found to refold to native conformation after denaturation and reductive cleavage of their disulfide bridges (for recent reviews, see refs. 22 and 23). In the present work we have shown that the kringle 4 fragment of human plasminogen also is capable of assuming the native, biologically active conformation after denaturation and reduction of its disulfide bonds, indicating that the fragment carries an intact folding unit of plasminogen. This observation adds further evidence in support of the idea that folding of large proteins occurs in subassemblies.

Refolding of kringle 4 is a remarkably well-directed process, as shown by our observation that of the possible 45 two-disulfide-bridge intermediates, only a single, biologically active variant appears, and of the 15 possible three-disulfide-bridge variants, only native kringle 4 is formed. Thus, folding of kringle 4 fragment appears to be better guided than is the folding of bovine pancreatic trypsin inhibitor. In the case of this small protein, which also contains three disulfide bonds, four different two-disulfide-bridge intermediates are significant and a three-disulfide-bridge variant with mismatched disulfide bonds also is formed (24).

Because the kringle 4 fragment contains only 10 residues more than the 79-residue segment common in all prothrombin, urokinase, and plasminogen kringles, it seems safe to suggest that it is the kringle structure that carries all of the information necessary for the autonomous folding of kringle 4. In view of these findings we suggest that the high degree of conservation of kringles reflects at least in part the importance of the conserved residues for the autonomous acquisition of the kringle structure, rather than for a very important, but as yet undetermined, function for the kringle regions, as has been suggested recently (12).

Residues Essential for the Autonomous Folding of Kringles. The different kringles of prothrombin, plasminogen, and urokinase, though retaining the same triple-loop architecture, diverged to bind different proteins or low molecular weight compounds. Therefore, if the amino acid sequences of kringles possessing different binding functions are compared, the residues involved directly in the diverse binding functions may show great variability, whereas the residues essential for the autonomous folding of kringles should be found to be unchanged in all or most of the kringles.

Comparison of the sequences of human plasminogen, urokinase, and prothrombin kringles (i.e., sequences of kringles with different binding specificities) is shown in Fig. 6. In this histogram, the ordinate indicates the maximal number of sequences in which the same (or chemically similar) amino acid is present at the given position. As eight sequences (sequences of the five plasminogen kringles, two prothrombin kringles, and a urokinase kringle) are compared a value of 8 indicates conservation of that residue in all kringles; conversely, a value of 1 indicates that all eight sequences contain a different amino acid at that sequence position.

As shown in Fig. 6, the conserved residues are clustered around Cys^{50}, Cys^{62}, and Cys^{74}, with the longest run of conserved residues being found around Cys^{50}. It seems likely that the high degree of conservation of these residues reflects the central role of these regions in kringle folding, a suggestion supported by our observation that the native Cys^{22} Cys^{62} and Cys^{50} Cys^{74} bridges are the first to form during refolding. Similarly, the variability of the residues neighboring the Cys^{1} and Cys^{79} residues harmonizes with our observation that the Cys^{1} Cys^{79} disulfide bridge is the last to form and is not essential for the maintenance of the biologically active conformation of kringle 4.

We suggest that the kringle architecture conserved in all prothrombin, urokinase, and plasminogen kringles serves as a scaffold and the different binding sites are accommodated mainly in peptide regions not reserved for the residues that determine folding. Such regions may be present in regions notable for their variability (Fig. 6). Our previous work has indeed shown that the residues essential for the binding of ω-aminocarboxylic acids by kringle 4 are located in these nonconservative parts of the inner loop, the carboxylate group of Asp^{56} and the guanidinium group of Arg^{70} being involved in binding the ammonium- and carboxylate-functions of ω-aminocarboxylic acids (11).

1. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. & Magnusson, S. (1978) in *Progress in Chemical Fibrinolysis and Thrombolysis*, eds. Davidson, J. F., Rowan, M. M. & Desnoyers, P. C. (Raven, New York), Vol. 3, pp. 191–209.
2. Magnusson, S., Sottrup-Jensen, L., Petersen, T. E. & Claeys, H. (1975) in *Proteases and Biological Control*, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), Vol. 2, pp. 123–149.
3. Walz, D. A., Hewett-Emmett, D. & Seegers, W. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1969–1972.
4. Günzler, W. A., Steffens, G. J., Ötting, F., Kim, S.-M.A., Frankus, E. & Flohe, L. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 1155–1165.
5. Wiman, B. & Collén, D. (1978) *Nature (London)* **272**, 549–550.
6. Jackson, C. M. & Nemerson, Y. (1980) *Annu. Rev. Biochem.* **49**, 765–811.
7. Dombrose, F. A., Gitel, S. N., Zawalich, K. & Jackson, C. M. (1979) *J. Biol. Chem.* **254**, 5027–5040.
8. Esmon, C. T. & Jackson, C. M. (1974) *J. Biol. Chem.* **249**, 7791–7797.
9. Wiman, B., Lijnen, H. R. & Collén, D. (1979) *Biochim. Biophys. Acta* **579**, 142–154.
10. Markus, G., Priore, R. L. & Wissler, F. C. (1979) *J. Biol. Chem.* **254**, 1211–1216.
11. Trexler, M., Váli, Zs. & Patthy, L. (1982) *J. Biol. Chem.* **257**, 7401–7406.
12. Young, C. L., Barker, W. C., Tomaselli, C. M. & Dayhoff, M. O. (1978) in *Atlas of Protein Sequence and Structure*, ed. Dayhoff, M. O. (National Biomedical Research Foundation, Silver Spring, Maryland), pp. 73–93.
13. Kurosky, A., Barnett, D. R., Lee, T.-H., Touchstone, B., Hay, R. E., Arnott, M. S., Bowman, B. H. & Fitch, W. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3388–3392.
14. Bloom, J. W. & Mann, K. G. (1979) *Biochemistry* **18**, 1957–1961.
15. Ploplis, V. A., Strickland, D. K. & Castellino, F. J. (1981) *Biochemistry* **20**, 15–21.
16. Castellino, F. J., Ploplis, V. A., Powell, J. R. & Strickland, D. K. (1981) *J. Biol. Chem.* **256**, 4778–4782.
17. Lerch, P.G., Rickli, E. E., Lergier, W. & Gillessen, D. (1980) *Eur. J. Biochem.* **107**, 7–13.
18. Váradi, A. & Patthy, L. (1981) *Biochem. Biophys. Res. Commun.* **103**, 97–102.
19. Seegers, W. H., Novoa, E., Walz, D. A., Andary, T. J. & Hassouna, H. I. (1976) *Thromb. Rès.* **8**, 83–97.
20. Váli, Zs. & Patthy, L. (1982) *J. Biol. Chem.* **257**, 2104–2110.
21. Saxena, V. P. & Wetlaufer, D. B. (1970) *Biochemistry* **9**, 5015–5023.
22. Wetlaufer, D. B. (1981) *Adv. Protein Chem.* **34**, 61–92.
23. Kim, P. S. & Baldwin, R. L. (1982) *Annu. Rev. Biochem.* **51**, 459–489.
24. Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol.* **33**, 231–297.